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Fusion protein predicted amino acid sequence of the first US avian pneumovirus isolate and lack of heterogeneity among other US isolates

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Abstract

Avian pneumovirus (APV) was first isolated from turkeys in the west-central US following emergence of turkey rhinotracheitis (TRT) during 1996. Subsequently, several APV isolates were obtained from the north-central US. Matrix (M) and fusion (F) protein genes of these isolates were examined for sequence heterogeneity and compared with European APV subtypes A and B. Among US isolates the M gene shared greater than 98% nucleotide sequence identity with only one nonsynonymous change occurring in a single US isolate. Although the F gene among US APV isolates shared 98% nucleotide sequence identity, nine conserved substitutions were detected in the predicted amino acid sequence. The predicted amino acid sequence of the US APV isolate's F protein had 72% sequence identity to the F protein of APV subtype A and 71% sequence identity to the F protein of APV subtype B. This compares with 83% sequence identity between the APV subtype A and B predicted amino acid sequences of the F protein. The US isolates were phylogenetically distinguishable from their European counterparts based on F gene nucleotide or predicted amino acid sequences. Lack of sequence heterogeneity among US APV subtypes indicates these viruses have maintained a relatively stable population since the first outbreak of TRT. Phylogenetic analysis of the F protein among APV isolates supports classification of US isolates as a new APV subtype C.

Keywords: Turkey rhinotracheitis virus; Fusion protein; Mononegavirales; Paramyxovirus; Viral evolution; Avian pathogen; Emerging diseases; Veterinary virology

Pneumoviruses are members of the family Paramyxoviridae which contain a nonsegmented negative-sense RNA genome of approximately 15 kb in length. Viruses related to avian pneumovirus (APV) include human, bovine, ovine and

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caprine respiratory syncytial viruses and pneumonia virus of mice. Although genome length is similar, pneumoviruses generally encode 10 genes, compared to six or seven in other paramyxoviruses. These include the nonstructural proteins (NS1 and NS2), nucleoprotein (N), phosphoprotein (P), matrix protein (M), small hydrophobic protein (SH), surface glycoprotein (G), fusion protein (F), second matrix protein (M2) and a viral RNA-dependent RNA polymerase (L). Although pneumoviruses have an F protein that promotes cell fusion, these viruses do not hemagglutinate, nor do they have neuraminidase activity in their G attachment protein. This is an important distinguishing characteristic from the other paramyxoviruses (reviewed in Collins et al., 1996).

Classification of European avian pneumovirus (APV) isolates was initially based on physical characterization of the virion (Collins et al., 1986; Collins and Gough, 1988), electrophoretic mobility of viral proteins (Ling and Pringle, 1988) and number of mRNA species detected in APV infected cells (Cavanagh and Barrett, 1988). Sequence information for the N (Li et al., 1996), P (Ling et al., 1995), M (Yu et al., 1992b; Randhawa et al., 1996a; Seal, 1998), F (Yu et al., 1991; Naylor et al., 1998), M2 (Yu et al., 1992a), SH (Ling et al., 1992), G (Ling et al., 1992; Juhasz and Easton, 1994) and L (Randhawa et al., 1996b) genes are now published for several APV isolates. The sequence in every case is most similar to other members of the *Pneumovirus* genus. The putative gene order of APV (3′N-P-M-F-M2-SH-G-L5′) is different from its mammalian counterparts (3′NS1-NS2-N-P-M-SH-G-F-M2-L5′), wherein the SH and G genes are located 5′ to the M2 gene (Ling et al., 1992). The extreme 3′ and 5′ ends of one European APV isolate's genome were determined which established that the NS1 and NS2 genes are absent in the avian viruses (Randhawa et al., 1997). This is different from their mammalian counterparts and along with a smaller L gene results in APV having a genome of only 13.3 kb (Randhawa et al., 1996b). Since APV has no NS1 or NS2 gene, but has a M2 gene with structural characteristics like other pneumoviruses, it has been suggested that it become the type virus of a new genus within the *Metapneumovirus* (Pringle, 1998).

The G protein of pneumoviruses is responsible for cell attachment and is a major antigenic determinant of this group. Based on G protein gene nucleotide and predicted amino acid sequences two APV subgroups, designated A and B, were identified (Juhasz and Easton, 1994). Group A viruses include isolates from the UK and France, while group B viruses include isolates from Spain, Italy and Hungary. However, subtype B viruses are now found in the UK (Naylor et al., 1998). The G proteins share approximately 99% identity among viruses in the same group. However, they are only 38% similar between viruses from the two APV subtypes. This correlates with earlier data demonstrating that various APV isolates were antigenically similar, but could be separated serologically into two distinct groups (Collins et al., 1993; Cook et al., 1993). This relationship was further confirmed by sequence analysis of the F protein gene (Naylor et al., 1998) and the more conserved M gene (Randhawa et al., 1996a). Differences in F and G proteins between APV subtypes could account for discrepancies using different antigens for serology (Eterradossi et al., 1992). Only one partial F gene sequence (accession no. D49957) is currently available for Japanese APV isolates (Tanaka et al., 1995, 1996). This sequence shares 100% nucleotide identity to the European subtype A isolate UK3B (data not shown). Isolates from Brazil conform to European subtype A (Dani et al., 1999), and turkeys in Chile were serologically positive by ELISA utilizing European APV antigens (Toro et al., 1998).

Avian pneumovirus causes turkey rhinotracheitis (TRT) and is associated with swollen head syndrome (SHS) of chickens which is usually accompanied by secondary bacterial infections that increase mortality. APV was first reported in South Africa during the early 1970s and was then isolated in Europe, Israel and Asia (reviewed in Jones, 1996; Alexander, 1997). During February, 1997 APV was officially isolated by the National Veterinary Services Laboratory (NVSL, APHIS, USDA) from commercial turkeys in Colorado (APV/CO) following an outbreak of TRT the previous year. During the first 10 months of the US outbreak it was not possible to detect virus serologically due to no cross-reactivity of the US APV isolates with reagents produced in Europe. An ELISA was developed by NVSL using inactivated purified APV/CO as an antigen and serological evidence of APV infection was subsequently also demonstrated in north-central US turkey flocks. In the US mortality due to APV infections has ranged from zero, to 30% when accompanied by bacterial infections, with condemnations due to airsacculitis (Kleven, 1997). Absence of serologic reactivity by APV/CO infected birds with APV subtype A and B isolates clearly demonstrated emergence of new strains of this virus previously considered exotic to North America.

The M protein gene is highly conserved among paramyxoviruses (Rima, 1989) and was used for initial molecular characterization of APV from the US (Seal, 1998). To further characterize the US APV isolates, the F protein gene was cloned and sequenced from the APV/CO isolate and for two APV isolates from Minnesota. The APV/CO and Minnesota isolates MN1a, MN1b and MN2a (provided by National Veterinary Services Laboratory, APHIS, USDA, Ames, IA) were propagated in quail tumor (QT) cells using standard techniques (Wyeth and Alexander, 1989; Alexander, 1997; Chiang et al., 1998). Following replication in QT cells, RNA was purified by guanidinium extraction (Chirgwin et al., 1979) of infected cells and ultracentrifuged through CsCl (Glisin et al., 1974). The 5′ region of conserved nucleotide sequence among APV M protein genes (Yu et al., 1992b; Randhawa et al., 1996a) were analyzed by the PRIMER2 (Scientific and Educational Software, Stateline, PA) computer program to obtain an oligonucleotide primer as described (Seal, 1998). Poly-A RNA was purified (Aviv and Leder, 1972) and cDNA was synthesized (Kotewicz et al., 1988; Life Technologies) followed by polymerase chain reaction (Belyavsky et al., 1989) utilizing an oligo-dT primer (GGGAGGCCCCT_{15}) with a conserved APV 5′ M gene primer (GGGGACAAGTIAAIATGGAGTC). Additionally, primers were synthesized from within the APV/CO M gene and used for 5′RACE (Frohman, 1993) to synthesize cDNA from the viral genomic RNA. Subsequently, an APV/CO 5′ F gene primer (GGGACAAGTGAAAATGTCTTGG) was used with the oligo-dT primer (Belyavsky et al., 1989) to synthesize full-length F genes for the APV isolates. Amplified products utilizing Elongase (Life Technologies/Gibco BRL, Grand Island, NY) for PCR were cloned using TA cloning systems (Mead et al., 1991; Seal, 1998) according to methods of the manufacturer (Invitrogen, San Diego, CA; Promega, Madison, WI). The ligated cDNA was introduced into *Escherichia coli* using standard transformation and plated on media with ampicillin (Hanahan, 1985).

Double-stranded sequencing (Sanger et al., 1977) with *Taq* polymerase (Applied Biosystems Inc.) and fluorescently labelled dideoxynucleotides was performed with an automated sequencer (Smith et al., 1986). Six independent APV M and F protein gene clones from two separate amplification and cloning reactions were sequenced for each US isolate. Nucleotide sequence editing, prediction of amino acid sequences, and protein computer structure predictions were completed using the DNASTAR (Madison, WI) and GeneWorks 2.3 programs (Intelligenetics, Mountain View, CA). Alignments were performed using the CLUSTALW method (Thompson et al., 1994). Nucleotide sequence analysis, including determination of synonymous and nonsynonymous substitutions (Nei and Gojobori, 1986), was completed using the Molecular Evolutionary Genetics Analysis system (MEGA; Kumar et al., 1993). Nucleotide sequence analyses were also completed in MULTICOMP with a 90 base pair sliding window (Reeves et al., 1994) using the algorithm of Li (1993) for determining synonymous and nonsynonymous substitutions. These data were prepared for graphical presentation using Excel98 (Microsoft, Seattle, WA). A statistical test for recombination among homologous nucleotide sequences was also completed (Sawyer, 1989). To determine relationships among APV isolates and how protein sequence information relates to current designations, analysis was performed by Phylogenetic Analysis Using Parsimony (PAUP; Swofford, 1998) following 2000 bootstrap replications (Hedges, 1992) with the bovine respiratory syncytial virus M (Samal and

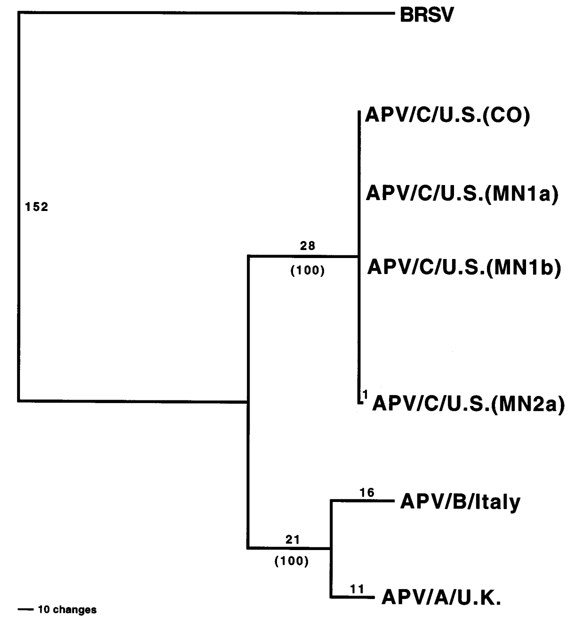

Fig. 1. Phylogenetic relationships among avian pneumoviruses based on matrix protein amino acid sequences. Following alignment a rooted phylogram was generated by maximum parsimony analysis using bovine respiratory syncytial virus (BRSV) as an outgroup. Absolute distances are listed above each branch with bootstrap confidence levels in parentheses below. APV represents avian pneumoviruses from the US and European subtypes A and B with geographic location.

Zamora, 1991) or F protein (Zamora and Samal, 1992) as an outgroup (Smith, 1994).

The M gene was cloned and sequenced for three APV isolates obtained from the north-central US, designated MN1a, MN1b and MN2a. Sequence analysis (Fig. 1) revealed that the viruses isolated from Minnesota, following initial isolation of APV during the Colorado outbreak are very homogeneous. In the M gene these viruses share a greater than 98% nucleotide sequence identity among one another. The few differences that occur are synonymous changes with the exception of the MN2a virus. One non-synonymous nucleotide change was present in the M gene that resulted in a single amino acid difference between the original CO isolate and the MN2a virus obtained 1 year later. As established previously (Seal, 1998), the US APV isolates can be distinguished phylogenetically from their European counterparts (Yu et al., 1992b; APV/A and Randhawa et al., 1996a; APV/B) as another distinct subtype (Fig. 1).

To further characterize these emergent viruses, the F protein was cloned, sequenced and compared phylogenetically to the European APV isolates (Naylor et al., 1998). Although the F protein genes among US APV isolates shared 98% nucleotide sequence identity, nine conserved substitutions were detected in the predicted amino acid sequence. The US APV/CO isolate's F protein predicted amino acid sequence shared 72% identity with the APV subtype A and 71% identity with the F protein of APV subtype B. This contrasts with the 83% identity shared between the F protein predicted amino acid sequences of APV subtypes A and B. A 67% identity in amino acid sequence of the F protein was shared among all three subtypes. The US viruses have an amino acid deletion in the F protein at position 528 relative to their European counterparts. Extensive variation occurs primarily in the amino-terminus from residues one through 20 and in the carboxy-terminus from residue 470 to the end of the F protein among APV subtypes (Fig. 2A). There are two potential N-glycosylation sites at amino acid positions 57 and 353 among the F proteins of all APV subtypes. The US APV isolates have a fusion protein cleavage site sequence of Arg–Lys–Ala–Arg/Phe–Val–Leu compared to Arg–Arg–Arg–Arg/Phe–Val–Leu for the A subtype and Arg–Lys–Lys–Arg/Phe–Val–Leu for the subtype B isolates (Fig. 2A).

The F protein mediates fusion of the viral membrane with the cell membrane that is cleaved into subunits F1 and F2 at an Arg–Lys rich region termed the connecting peptide (Collins et al., 1996). As reported for APV subtype A and B isolates, the most conserved portion of the F1 is the N-terminal region which is presumed to be involved with membrane fusion (Naylor et al., 1998). The Arg, Phe, Gly, and Gly at positions 102, 103, 106 and 111, respectively, are structurally important to paramyxovirus F proteins (Horvath and Lamb, 1992). These amino acids are conserved among all the APV subtypes relative to other paramyxoviruses. The F1 is more highly conserved than the F2 with the exception of the highly variable transmembrane domain and cytoplasmic tail of F1. This is similar to human

A

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APV/C(CO)        .SW.VV...V.LAT. T.GLE.S.L...Y.. .....L......... ...T..V.D...L.. T......R...E... ...E.........L.   90
APV/C(MN2a)      .SW.VV...V.LAT. T.GLE.S.L...... .....L......... ...T..V.D...L.. T......R...E... ...E.........L.   90
APV/B(Hungary)   .YL.LL.IIY.VVGA ..K.....S...... ............... .....K......... .......S...S..Q ...Q..R......IT   90
APV/B(Italy)     .YL.LL.IIY.VVGA ..K.....S...... ............... ............... .......S...S..Q ...Q..R......IT   90
APV/A(UK3B)      .DVRIC...F.ISN. .SC.....N...... ............... ............... .......D...V... ...R.........V.   90

Consensus        M..K..LLL.L...P SG.IQETY.EESCST VTRGYKSVLRTGWYT NVFNLEIGNVENITC NDGPSLI.TEL.LTK NAL.ELKTVSADQ.A   90

APV/C(CO)        ..A..M....A.... ............... ...I..........A ...G...K....... ............... .D...........R.  180
APV/C(MN2a)      ..A..M....A.... ............... ...I..........A ...G...K....... ............... .D...........K.  180
APV/B(Hungary)   ..N..L.H..K.... .........T..... ............... ...L...S....... ......I........ ...............  180
APV/B(Italy)     ..N..L.H..K.... .........T..... ............... ...L...S....... ......I........ ...............  180
APV/A(UK3B)      ..S.LS...RR.... ............... ............... ...N...N....... ............... ...............  180

Consensus        KE.RI.SPRK.RFVL GAIALGVATAAAVTA GVALAKTIRLEGEVK AIK.ALR.TNEAVST LGNGVRVLATAVNDL KEFISKKLTPAINQN  180

APV/C(CO)        ..D.S.L...V.... Y............N ....P.I........ .....VSN....... .N...E......... .........SS...I  270
APV/C(MN2a)      ..D.S.L...V.... Y............N ....P.I........ .....VSN....... .N...E......... .........SS....  270
APV/B(Hungary)   .......R....... ............... ............... ...K........... ...........K... ...............  270
APV/B(Italy)     .......R....... ............... ............... ...K........... ............... ...............  270
APV/A(UK3B)      ............... ............... ..............D ............... ............... ........D......  270

Consensus        KCNIADIKMAISFGQ NNRRFLNVVRQFSDS AGITSAVSLDLMTDA ELVRAINRMPTSSGQ ISLMLNNRAMVRRKG FGILIGVYGGTVVYM  270

APV/C(CO)        .........D....K .K.....SGKD.... ...........Q... ..V.....E.....S .H............K .S.E..R....T...  360
APV/C(MN2a)      .........D....K .K.....SGKD.... ...........QD.. ..V.....E.....S .H............K .S.E..R....T...  360
APV/B(Hungary)   ............... ........H.RES.. ............... ............... ..............S ......Q.....T..  360
APV/B(Italy)     ............... ........H.RES.. ............... ............... ..............S ......H.....T..  360
APV/A(UK3B)      ............... ........K.K.... .I............. .......K....... ..............L ......Y........  360

Consensus        VQLPIFGVIETPCWR VVAAPLCR.E.GNYA CLLREDQGWYCTNAG STAYYPNEDDCEVRD DYVFCDTAAGINVA. EVEQCN.NISTSKYP  360

APV/C(CO)        .........I..... S...A..A..D.M.. ..........RP... ..SY.S.QD...V.. ..........E...H ....K..SS..D..E  450
APV/C(MN2a)      .........I..... S...A..A..D.... ..........RP... ..SY.S.QD...V.. ..........E...H ....K..SS..D..E  450
APV/B(Hungary)   ............... ............... .............N. ............... ...I........... .............L.  450
APV/B(Italy)     ............... ............... .............N. ............... ...I........... .............L.  450
APV/A(UK3B)      ............... ...........S... ............... ............... ............... ...............  450

Consensus        CKVSTGRHPVSMVAL TPLGGLVSCYEGVSC SIGSNKVGIIKQLGK GCTHIPNNEADTITI DNTVYQLSKVVGEQR TIKGAPVVNNFNPIL  450

APV/C(CO)        .......I....... ..E...N...Q..KI ..SIE.G...FV.V. .LI.L.MLAAVGVGV FF.......A..FPM EMN-.VN.K.F.P  537
APV/C(MN2a)      .......I....... ..E...N...Q..KI ..STE.G...FV.V. .LI.L.VLGAVGVGV FF.......A..FPM EMN-.VN.K.F.P  537
APV/B(Hungary)   ............... .....K......... ..IEV...I.A.L.. TI..V.SMSIIVGIA ..........ASNG. .K...Q.......  538
APV/B(Italy)     ............... .....K......... ..IEV...I.A.L.. TI..V.SMLIIVGIA ..........TSNG. .K...Q.......  538
APV/A(UK3B)      ............... .I.R........... .GADA..K..I.... .V..I.GIFFLLAVI ..CSRV..T...HD. .A...H.S.A.V.  538

Consensus        FPEDQFNVALDQVFE SVDKSQDLIDKSNDL LD...KSNAG.AIAI V.LV.L......... YYVVKKRKAKPK..Y P.TTG.SNMGYIS  538
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B

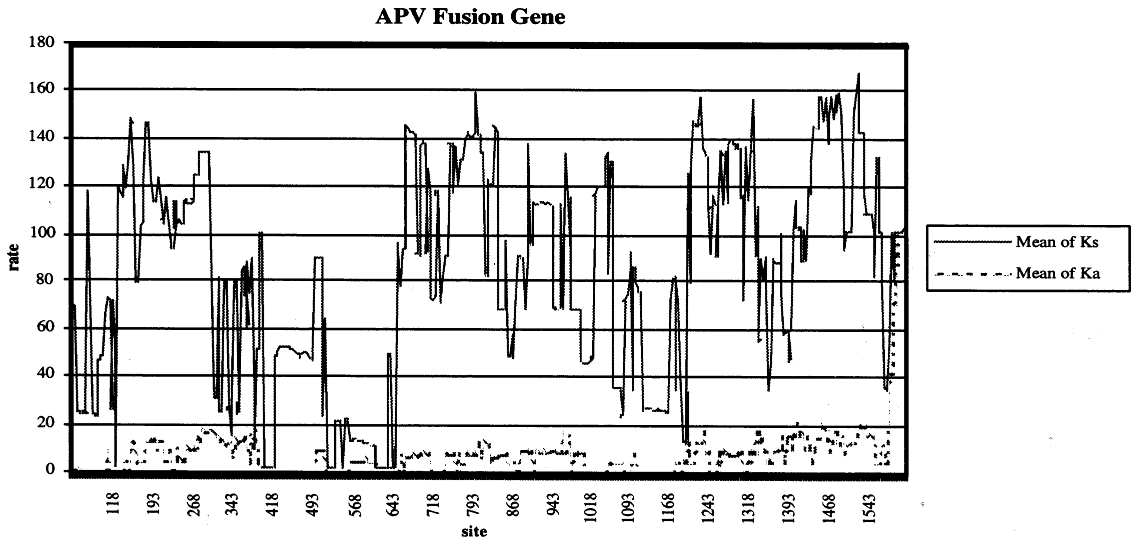

Fig. 2.

respiratory syncytial virus (HRSV), wherein these regions vary between subtypes, but within subtypes they are highly conserved (Plows and Pringle, 1995).

Cellular proteases that recognize the sequence Arg–X–Arg/Lys–Arg (Hosaka et al., 1991) are required to cleave paramyxovirus fusion proteins (Nagai, 1995). The US isolates differ from the European APV subtypes in that they have Ala at position 101 instead of a basic amino acid (Naylor et al., 1998). The Ala present in the F protein cleavage site of US isolates rather than Arg or Lys present among European strains may affect relative virulence among these viruses. This variation may be important in modulating efficiency of cleavage or may affect the range of host proteases capable of cleaving the F protein. Since severe disease is observed primarily when APV is associated with bacterial infections (Majo et al., 1997), it is possible that bacterial proteases (Akaike et al., 1989) may also play a role in APV fusion protein cleavage.

Overall 57% nucleotide sequence identity was shared among the three subtypes in the F gene open reading frame. The US viruses shared 67% identity with subtype A and 61% with subtype B. Sequence variation occurs throughout the F protein gene of all three subtypes, with synonymous changes predominating (Fig. 2B). The nonsynonymous to synonymous base change ratio was 0.77 when comparing the US isolates relative to the European subtype A and B viruses. This contrasts with a ratio of 0.51 when determining this ratio for the F gene of the APV subtype A and B isolates. The amino-terminus (residues 1 through 24) and carboxy-terminus (from residue 465) of the F protein are highly variable relative to the interior of the protein. The nonsynonymous to synonymous base ratio for the amino-terminus F gene coding region is 0.68 when comparing the US subtype viruses relative to the A and B subtypes, while the ratio is only 0.50 when comparing the A and B subtypes to each other. This contrasts with the carboxy-terminus coding region wherein the nonsynonymous to synonymous base ratio is 2.0 when determining this value for the US virus relative to the A and B subtypes. This ratio is 1.5 when comparing the A and B subtypes of APV.

The overall ratio of nonsynonymous to synonymous bases in the F gene among APV isolates is less than one and indicates these viruses have been undergoing purifying selection resulting in disadvantageous nonsynonymous mutations being eliminated (Kimura, 1977). However, certain portions of the F gene have a greater number of nonsynonymous base changes, illustrated by increased variability in the amino- and carboxy-terminal portions of the F protein (Fig. 2A). This is similar to other viral systems, wherein certain regions of a gene may have a greater rate of nonsynonymous substitutions relative to the overall rate for the entire gene (Ina and Gojobori, 1994; Seibert et al., 1995). The hypervariable F protein regions of APV subtypes are comparable to regions of F proteins that vary between the two HRSV subtypes (Johnson and Collins, 1988).

The F protein cleavage site sequences are different for each APV subtype (Fig. 2A). The first basic amino acid (Arg) at residue 99 in the cleavage site sequence is shared by all three subtypes. However, the US viruses use an AGA codon while European viruses utilize an AGG. Although the Lys at position 100 of the F protein is shared by the US viruses with their European subtype B counterparts, the codon usage is not identical. The US isolates have an AAA codon while the subtype B viruses utilize an AAG. The subtype A viruses have an AGA for Arg at position 100 rather than another AGG. Residue 101 of the F protein differs for all three viruses with GCC coding for an Ala among US viruses. This is significantly different from the AAG coding for Lys among subtype B viruses which represents a

Fig. 2. Comparative amino acid sequence alignment of fusion proteins along with fusion protein gene heterogeneity among avian pneumovirus isolates. (A) Sequences were aligned and amino acid differences denoted by the single-letter code with consensus below. The cleavage site is underlined. (B) Sliding window analysis of synonymous and nonsynonymous base substitutions in the fusion protein gene among APV isolates. Solid line represents the rate of synonymous (*K*s) base substitutions and the dashed line represents the rate of nonsynonymous (*K*a) base substitutions in a 90 base pair window.

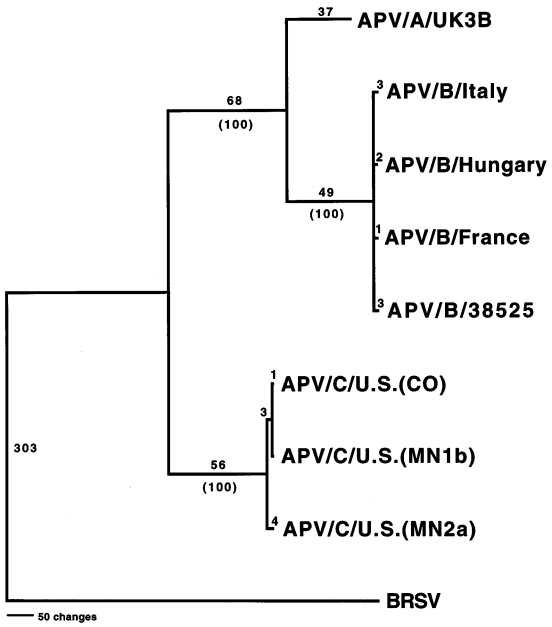

Fig. 3. Phylogenetic relationships among avian pneumoviruses based on fusion protein amino acid sequences. Following alignment, a rooted phylogram was generated by maximum parsimony analysis using bovine respiratory syncytial virus (BRSV) as an outgroup. Absolute distances are listed above each branch with bootstrap confidence levels in parentheses below. APV represents avian pneumoviruses from the US and European subtypes A and B with geographic location.

repeat of the previous codon. This contrasts with the CGU coding for Arg among subtype A viruses at residue 101 of the F protein. The final Arg of the cleavage site at residue 102 is shared by all three APV subtypes. However, the US viruses utilize a CGG, while the subtype A viruses have an AGA codon and the subtype B isolates use the AGG sequence. The F protein cleavage site sequence varies among all three APV subtypes and the codon usage is very heterogenous but consistent with the three distinct lineages. Finally, there was variation in the number of nucleotides in both the 5′ and 3′ untranslated regions of the F gene when comparing the US viruses to the subtype A and B viruses. Therefore, APV does not require the genome to contain a number of nucleotides divisible by six (Randhawa et al., 1997).

Phylogenetic analysis of the F protein amino acid sequences demonstrates that the US viruses form a distinct subtype from the European viruses, that is supported by high bootstrap confidence levels (Fig. 3). A relationship with identical topology was obtained when analyzing nucleotide sequences utilizing distance matrix (Saitou and Nei, 1987) or parsimony (Swofford, 1998) methods (data not shown). This is similar to results obtained using the M protein predicted amino acid or M gene nucleotide sequences (Seal, 1998).

Based on nucleotide and predicted amino acid sequences, two APV subtypes, designated A and B, have been reported (Juhasz and Easton, 1994; Randhawa et al., 1996a; Naylor et al., 1998). This correlates with the finding that European APV isolates could be differentiated serologically into two separate groups (Collins et al., 1993; Cook et al., 1993). The APV isolates from the US are serologically distinct from European APV subtypes A and B (Kleven, 1997). This relationship is supported by phylogenetic analysis of the M protein gene (Seal, 1998) and corroborated by M and F gene sequences among geographically separate US isolates reported herein. These results confirm emergence of unique APV subtype C isolates in the US distinct from European viruses.

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